



## Exercise ameliorates chronic inflammatory response induced by high-fat diet via Sestrin2 in an Nrf2-dependent manner



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### ABSTRACT

Chronic inflammation is a major contributor to the development of metabolic disorders and is commonly seen in studies of diet-induced obesity in humans and rodents. Exercise has been shown to have anti-inflammatory properties, though the exact mechanisms are still not fully understood. Sestrins and Nrf2 are of interest to researchers as they are known to protect against inflammation and oxidative stress. In this study, we aim to explore the interconnection between Sestrin2 (SESN2) and Nrf2 and their roles in exercise benefits on chronic inflammation. Our data showed that SESN2 knockout aggravated the abnormalities of body weight, fat mass, and serum lipid that were induced by a high-fat diet (HFD), and a concomitant increase of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in both serum and skeletal muscle. Notably, exercise was found to reverse these changes, and SESN2 was found to be necessary for exercise to reduce the inflammatory response in skeletal muscles, though not in serum. Immunoprecipitation and bioinformatics prediction experiments further revealed that SESN2 directly binds to Nrf2, indicating a protein-protein interaction between the two. Furthermore, our data demonstrated that SESN2 protein is necessary for exercise-induced effects on Nrf2 pathway in HFD-fed mice, and Nrf2 protein is necessary to enable SESN2 to reduce the inflammation caused by palmitic acid (PA)+ oleic acid (OA) treatment in vitro. Our findings indicate that exercise mitigates chronic inflammation induced by HFD through SESN2 in an Nrf2-dependent manner. Our study reveals a novel molecular mechanism whereby the SESN2/Nrf2 pathway mediates the positive impact of exercise on chronic inflammation.

### 1. Introduction

We have introduced numerous false inflammatory triggers in our lifestyle, driving us to a state of chronic inflammation which is linked to the development of metabolic diseases [1,2]. This chronic inflammation has been found to be caused by obesity due to overeating and physical inactivity, with inflammatory cells accumulating in fat and other tissues

[3]. According to several human and rodent studies, skeletal muscle, as an organ closely related to exercise, can secrete C reactive protein, interleukin 6 (IL-6) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) to induce chronic inflammation in sarcopenia, obesity or insulin resistance [4–7]. Excessive intake of a high-fat diet (HFD) has been identified as the primary factor leading to chronic inflammation [8,9]. And long-term HFD has been observed to activate the nuclear factor-kappaB (NF- $\kappa$ B)

**Abbreviations:** SESNs, Sestrins; SESN2, Sestrin2; HFD, high-fat diet; PA, palmitic acid; OA, oleic acid; IL-6, interleukin 6; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; NF- $\kappa$ B, nuclear factor- kappaB; HIIT, high-intensity interval training; NASH, non-alcoholic steatohepatitis; SESN1, Sestrin1; SESN3, Sestrin3; NAFLD, non-alcoholic fatty liver disease; LPS, lipopolysaccharide; Nrf2/Nfe2l2, nuclear factor erythroid 2-related factor; Keap1, kelch-like ECH-associated protein 1; WT, wild type; NC, normal chow; HC, high-fat diet chow; HE, high-fat diet chow plus exercise; TC, total cholesterol; TG, triglycerides; IL-1 $\beta$ , interleukin-1 $\beta$ ; PVDF, polyvinylidene difluoride; HO-1, heme oxygenase-1; HRP, horseradish peroxidase; MOI, multiplicity of infection; ORO, oil red O.

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pathway and macrophage infiltration in the skeletal muscles of C57BL/6 J mice, which was reduced through eight weeks of aerobic exercise [9]. In addition, high-intensity interval training (HIIT) has been found to effectively reduce hepatic inflammation, particularly the accumulation of hepatic monocyte-derived inflammatory macrophages during non-alcoholic steatohepatitis (NASH) induced by a high-fat and high-carbohydrate diet for 6 weeks [10]. This is further supported by evidences that regular exercise can have anti-inflammatory effects [3,4,9–11]. However, the underlying mechanisms of how exercise can help to alleviate chronic inflammation induced by a high-fat diet in skeletal muscle are still not fully understood.

Sestrins (SESNs) have been identified as a family of highly conserved stress-inducible proteins and play protective roles in most physiological and pathological conditions mainly through the regulation of oxidative stress, inflammation, autophagy, endoplasmic reticulum stress, and metabolic homeostasis [12,13]. There are three isoforms in vertebrates including Sestrin1 (SESN1), Sestrin2 (SESN2) and Sestrin3 (SESN3) [4,13]. Several studies have demonstrated the positive effects of SESN2 in various conditions. For instance, SESN2 inhibits lipogenesis and inflammation in non-alcoholic fatty liver disease (NAFLD) induced by HFD via AMPK/mTOR pathway [14]. Additionally, overexpression of SESN2 could inhibit the expression of proinflammatory cytokines induced by lipopolysaccharide (LPS), contributing to the improvement of inflammatory diseases [15]. Our previous study further showed that overexpression of SESN2 reduced lipid biosynthesis through inhibition of JNK in palmitate-treated C2C12 [16]. Recent evidence indicates that SESNs could mimic the physiological effects of exercise [17], suggesting that SESN2 could be evolutionarily conserved mediators of exercise benefits. Our previous data revealed that regular exercise improves mitochondrial dysfunction of sarcopenia and abnormal glucose-lipid metabolism induced-HFD, and a concomitant increase of SESN2 protein expression in the skeletal muscle [4,16,18,19]. Additionally, it has been demonstrated that the acute aerobic exercise induces autophagy and increases the protein expression of SESN2 in the skeletal muscle [20]. Although its underlying mechanism is still elusive, SESN2 could be evolutionarily conserved mediators of exercise benefits.

Nuclear factor erythroid 2-related factor (Nrf2) is a transcription factor that plays an important role in cell defense against oxidative stress and inflammation [21,22]. Generally, Nrf2 exerts its functions through the interaction with Kelch-like ECH-associated protein 1 (Keap1) [21]. Under normal conditions, Nrf2 binds to Keap1 to regulate proteasomal degradation. However, when oxidative stress occurs, the interaction between Nrf2 and Keap1 is disrupted, allowing Nrf2 to translocate to the nucleus and control a variety of downstream antioxidant and anti-inflammatory genes [21]. It has been reported that knockout of Nrf2 increases the expression of proinflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-8) induced by LPS and increases the TLR4, p-IkBa/IkB $\alpha$ , and p-p65/p65 proteins expression in goat endometrial epithelial cells [23]. In addition, red ginseng-derived saponin fraction suppresses the obesity-induced inflammatory responses via Nrf2-HO-1 pathway in adipocyte-macrophage co-culture system [24]. Further evidence of exercise promoting Nrf2 expression in skeletal muscle has been demonstrated [25,26]. Studies have shown that long-term exercise intervention improves skeletal muscle function and restores the sarcopenia-like phenotype in C57BL/6 J mice, which is accompanied by an increase in the mRNA level of Nrf2 [25]. Additionally, it has been demonstrated that 4-week of voluntary wheel running increases Nrf2 translocation into nuclei, as well as Nrf2 DNA-binding activity in association with increased p62 phosphorylation (Ser351) in mouse oxidative skeletal muscle [26]. Collectively, there evidences demonstrate that both SESN2 and Nrf2 are regulated by exercise, and have powerful anti-inflammatory effects. However, the relationship between SESN2 and Nrf2 remains unclear. In this study, we employed two distinct chronic inflammation models, one induced by long-term HFD feeding in vivo and another, in vitro, by treatment with palmitic acid (PA) + oleic acid (OA), to investigate the correlation and potential mechanism between

SESN2 and Nrf2 in the exercise-mediated benefits of chronic inflammation.

## 2. Materials and methods

### 2.1. Animals

Male C57BL/6 J mice (4-week-old, wild type, WT) were purchased from SPF Biotechnology Co., Ltd. (Beijing, China). Male SESN2<sup>-/-</sup> mice (4-week-old) were established at the Model Animal Research Centre of Nanjing University, China (GemPharmatech Co., Ltd). Mice were housed in a temperature-controlled environment, with 12:12 h light/dark cycle, free access to food and water. WT mice were randomly divided into normal chow control group (WT-Ctrl) and high-fat diet (HFD) group (WT-HFD). And SESN2<sup>-/-</sup> mice (SESN2<sup>-/-</sup>-HFD) were also fed HFD for 6 weeks. After 6-week, mice from HFD groups (WT-HFD and SESN2<sup>-/-</sup>-HFD) were randomly subdivided into sedentary groups (WT-HC and SESN2<sup>-/-</sup>-HC) and exercise groups (WT-HE and SESN2<sup>-/-</sup>-HE). Mice from WT-HE and SESN2<sup>-/-</sup>-HE groups underwent the exercise training on a motor-driven rodent treadmill for 5 days per week for a total of 6 weeks. The mice initially ran at the intensity of 50 % VO<sub>2max</sub> for 20 min/day during the first week; thereafter, the running intensity and time were increased to 75 % VO<sub>2max</sub> (12 m/min) for 60 min/day. During the exercise training period, mice from HFD groups continued to eat a high-fat diet (45 % calories from fat, #D12451, Research Diets). Body weight of mice was determined weekly. The ratio of body weight gain is calculated by the difference of body weight between the latter and the previous week/the body weight of the previous week. After the desired length of exercise training, the body composition including lean mass and fat mass were monitored with an ImpediVET analyzer (ImpediMed). Mice were then euthanized, blood, the vastus lateralis muscle and liver were harvested for analysis. All animal protocols were approved by the Tianjin Medical University Animal Care and Use Committee (approval number: SYXK-2019-0004) under the guidelines of the animal welfare and experimental protocol of the Chinese Academy of Sciences.

### 2.2. Serum profiles analysis

Serum was obtained by centrifugation at 3000 rpm for 30 min at 4 °C. The total cholesterol (TC), triglycerides (TG), IL-6, interleukin-1 $\beta$  (IL-1 $\beta$ ) and TNF- $\alpha$  in serum were enzymatically determined using individual commercial kits (Nanjing Jiancheng, Nanjing, China and Shanghai Meilian, Shanghai, China). All experiments were run in triplicate.

### 2.3. Western blot analysis

Total protein from tissues/cells was extracted in freshly prepared NP-40 lysis buffer (150 mM sodium chloride, 1.0 % NP-40, 50 mM Tris [pH 8.0], protease inhibitor cocktail, and phosphatase inhibitor cocktail). Nuclear and cytoplasmic fractions from tissues/cells were extracted by nuclear/cytoplasmic fractionation Kit (AmyJet, AKR-171). Equal amounts of protein were separated by SDS-PAGE at 110 V and transferred to polyvinylidene difluoride (PVDF, 0.45  $\mu$ m) membranes at 300 mA for 2 h. The membranes were blocked and incubated overnight at 4 °C with primary antibodies against IL-6 (ABclonal, A0286), IL-1 $\beta$  (ABclonal, A16288), TNF- $\alpha$  (ABclonal, A11534), SESN1 (Proteintech, 21668-1-AP), SESN2 (Abcam, ab178518), SESN3 (Proteintech, 11431-2-AP), nuclear factor erythroid derived 2-like 2 (Nrf2, ABclonal, A1244), kelch-like ECH-associated protein 1 (Keap1, CST, #8047), heme oxygenase-1 (HO-1, Abcam, ab13243),  $\beta$ -tubulin (CST, #2146), LaminB1 (CST, #13435) and GAPDH (ABclonal, A19056). The target proteins were detected with chemiluminescent horseradish peroxidase (HRP, Proteintech, SA00001-2). Specific bands densitometry was analyzed using Quantity-one software. All results are representative of three independent experiments.

## 2.4. Bioinformatics prediction of protein-protein interactions

The STRING database (<https://string-db.org/>) is used to predict protein-protein interactions. We choose “Multiple Proteins”, import target proteins in “List of Names” and set “*Mus musculus*” in “Organisms”. And we restrict the “max number of interactors” not exceed 10 interactions. Network nodes represent proteins. And splice isoforms or post-translational modifications are collapsed, i.e. each node represents all the proteins produced by a single, protein-coding gene locus. The colored nodes represent query proteins and first shell of interactors. The white nodes represent second shell of interactors. The empty nodes represent proteins of unknown 3D structure. The filled nodes represent some 3D structure is known or predicted. Edges represent protein-protein associations. And those associations are meant to be specific and meaningful, i.e. proteins jointly contribute to a shared function; this does not necessarily mean they are physically binding to each other. The annotation of different colored edges is shown in Fig. 3.

## 2.5. Immunoprecipitation

Anti-Nrf2 primary antibody was cross-linked to Dynabeads® Protein A (Invitrogen, 10008D) according to the manufacturer's protocol. Tissue lysates were precleared with IgG Dynabeads® Protein A for 10 min and then incubated with Nrf2-Dynabeads. Nrf2 immunoprecipitated complexes were washed five times with washing buffer. Proteins were eluted by boiling in loading buffer and then processed for western blot analysis.

## 2.6. Cell culture and treatments

C2C12 cell (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % FBS in a humidified atmosphere with 5 % CO<sub>2</sub> at 37 °C. Cells were differentiated into myotubes in DMEM with 2 % horse serum for 6 days. For overexpression of SESN2, adenovirus experiments, C2C12 were infected by SESN2 adenoviruses (Ad-SESN2) using 100 multiplicity of infection (MOI) and harvested after 48 h. For silencing Nrf2 expression, C2C12 were incubated in ML-385 (20 µM) for 24 h. To establish in vitro model of lipid metabolic disorder, C2C12 myotubes were incubated with palmitic acid (PA, 0.8 mM) and oleic acid (OA, 1.6 mM) for 24 h. All experiments were performed in triplicate.

## 2.7. Oil red O (ORO) staining

ORO staining was used to detect lipid droplets accumulation in PA + OA-treated C2C12 myotubes. Myotubes were washed with PBS, fixed in 4 % paraformaldehyde in PBS for 10 min, and then rinsed with PBS. And then the myotubes were stained with ORO (in isopropanol) for 5 min, rinsed in 60 % isopropanol. After washed by PBS, ORO images were taken by using light microscope (Olympus, Tokyo, Japan).

## 2.8. Statistical analysis

All data are presented as means ± SEM. We performed the unpaired Student's 2-tailed *t*-test for comparison of 2 experimental groups and one-way or two-way ANOVA in >2 groups followed by Tukey's post hoc test. *P* < 0.05 was considered to represent a significant difference. Statistical analyses were performed using SPSS 22.1.

## 3. Results

### 3.1. SESN2 knockout aggravates the abnormality of body weight and serum lipid induced by HFD

To examine the effects of high-fat diet, WT and SESN2<sup>-/-</sup> mice were fed HFD for 6 weeks. Our results showed that the body weight significantly increased starting from 4-week in WT-HFD mice when compared

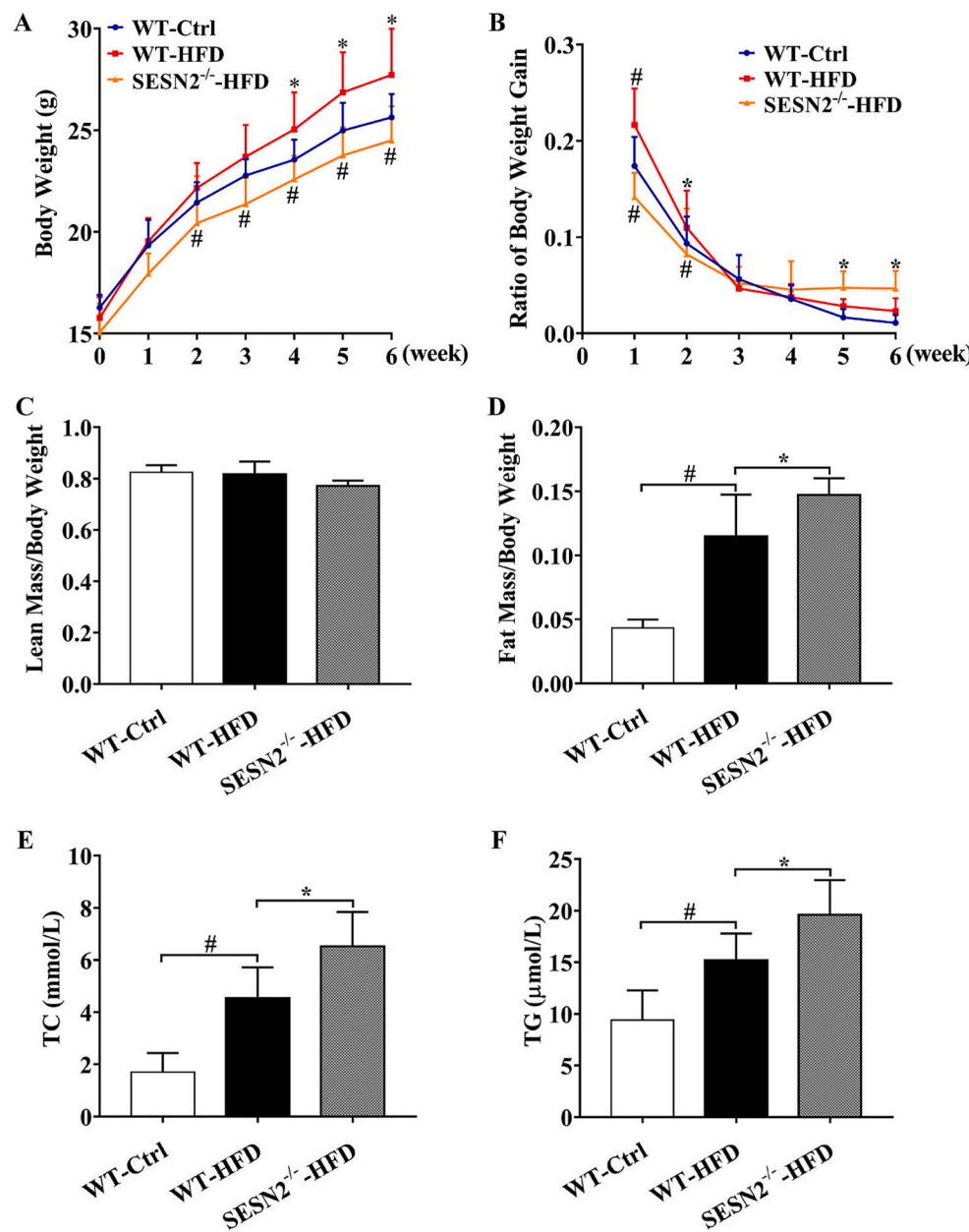
with the WT-Ctrl group (Fig. 1A). However, compared with the WT-HFD group, the body weight was significantly decreased starting from 2-week HFD in SESN2<sup>-/-</sup>-HFD mice (Fig. 1A). To eliminate the influence of genotype, we further detected the ratio of body weight gain after 6-week HFD. We found that there was a significant increase within 1–2 weeks and no significant difference within 3–6 weeks after HFD in the ratio of body weight gain in WT mice (Fig. 1B). While the ratio was significantly reduced within 1–2 weeks, not significantly changed within 3–4 weeks and significantly increased within 5–6 weeks after HFD in SESN2<sup>-/-</sup> mice (Fig. 1B). These results suggest that SESN2 deficiency exacerbates the body weight gain induced by HFD. Additionally, the body composition (lean mass and fat mass) and serum lipid profiles (TC and TG) were further examined in WT and SESN2<sup>-/-</sup> mice. Despite the lack of a notable difference in lean mass between WT and SESN2<sup>-/-</sup> mice after 6 weeks of HFD (Fig. 1C), the fat mass, TC and TG levels all exhibited a significant increase, with SESN2 deficiency exacerbating these trends (Fig. 1D-F).

### 3.2. Aerobic exercise ameliorated inflammation induced by HFD and SESN2 knockout

To examine the effects of regular exercise, WT and SESN2<sup>-/-</sup> mice were subjected to a 6-week aerobic exercise after 6-week HFD treatment. We found that the body weight significantly reduced after the long-term aerobic exercise both in WT and SESN2<sup>-/-</sup> mice (Fig. 2A). Notably, compared with WT-HC group, the body weight of WT-HE significantly decreased starting from 8-week (Fig. 2A); while compared with SESN2<sup>-/-</sup>-HC group, the body weight of SESN2<sup>-/-</sup>-HE significantly decreased starting from 10-week (Fig. 2A). The above data demonstrated that SESN2 deficiency limited the beneficial responses of exercise on body weight after HFD treatment. Additionally, while the body weight significantly decreased in the SESN2<sup>-/-</sup>-HC mice when compared with the WT-HC group (Fig. 2A), the fat mass/body weight was still increased (Fig. 2B) after HFD in SESN2<sup>-/-</sup> mice, indicating that SESN2 knockout mice are more sensitive to high-fat diet.

In addition, we observed that the fat mass/body weight significantly decreased in the SESN2<sup>-/-</sup>-HE mice compared to the SESN2<sup>-/-</sup>-HC group (Fig. 2B), suggesting that aerobic exercise can reduce the fat mass in SESN2 knockout mice. We further measured the inflammatory factors including TNF-α, IL-1β and IL-6 in serum after 6-week exercise training. Our data showed that long-term HFD led to a significant increase in the aforementioned inflammatory factors in serum, which was reversed by aerobic exercise in WT mice (Fig. 2C-E). However, compared to the WT-HC group, the inflammatory factors including TNF-α, IL-1β and IL-6 were significantly increased in serum of mice from SESN2<sup>-/-</sup>-HC group (Fig. 2C-E), demonstrating that knockout of SESN2 stimulated the production of high levels of systemic inflammatory response induced by HFD.

In addition, aerobic exercise can decrease the content of TNF-α, IL-1β and IL-6 in serum of SESN2<sup>-/-</sup>-HE mice when compared to SESN2<sup>-/-</sup>-HC mice (Fig. 2C-E), suggesting that SESN2 is not essential in the systemic inflammatory response. Aerobic exercise elicits many adaptations in skeletal muscles [4,27]. Therefore, we continued examining the protein expression of TNF-α, IL-1β and IL-6 in skeletal muscle. Consistent with the trend of serum, long-term HFD led to a significant increase of TNF-α, IL-1β and IL-6 protein in skeletal muscles, which was reversed by aerobic exercise in WT mice (Fig. 2F-I). Interestingly, SESN2 knockout also caused a significant increase of TNF-α and IL-1β protein in skeletal muscles after HFD (Fig. 2F-I). However, there was no significant difference between SESN2<sup>-/-</sup>-HC and SESN2<sup>-/-</sup>-HE groups with respect to these proteins of inflammatory factors, indicating that SESN2 is required for the exercise-induced reduction of inflammatory response in skeletal muscles (Fig. 2F-I).



**Fig. 1.** SESN2 knockout aggravates the abnormality of body weight and serum lipid induced by HFD. (A) Body weight and (B) the ratio of body weight gain were examined after 6-week HFD feeding. \*  $P < 0.05$  WT-HFD vs. WT-Ctrl; #  $P < 0.05$  SESN2<sup>-/-</sup>-HFD vs. WT-HFD. (C) Lean mass and (D) fat mass were evaluated and normalized to body weight. (E) TC and (F) TG were measured in WT and SESN2<sup>-/-</sup> mice. Data are presented as means  $\pm$  SEM ( $n = 6$ ). \*  $P < 0.05$ ; #  $P < 0.01$ . HFD, high-fat diet; WT, wild type; TC, total cholesterol; TG, triglycerides.

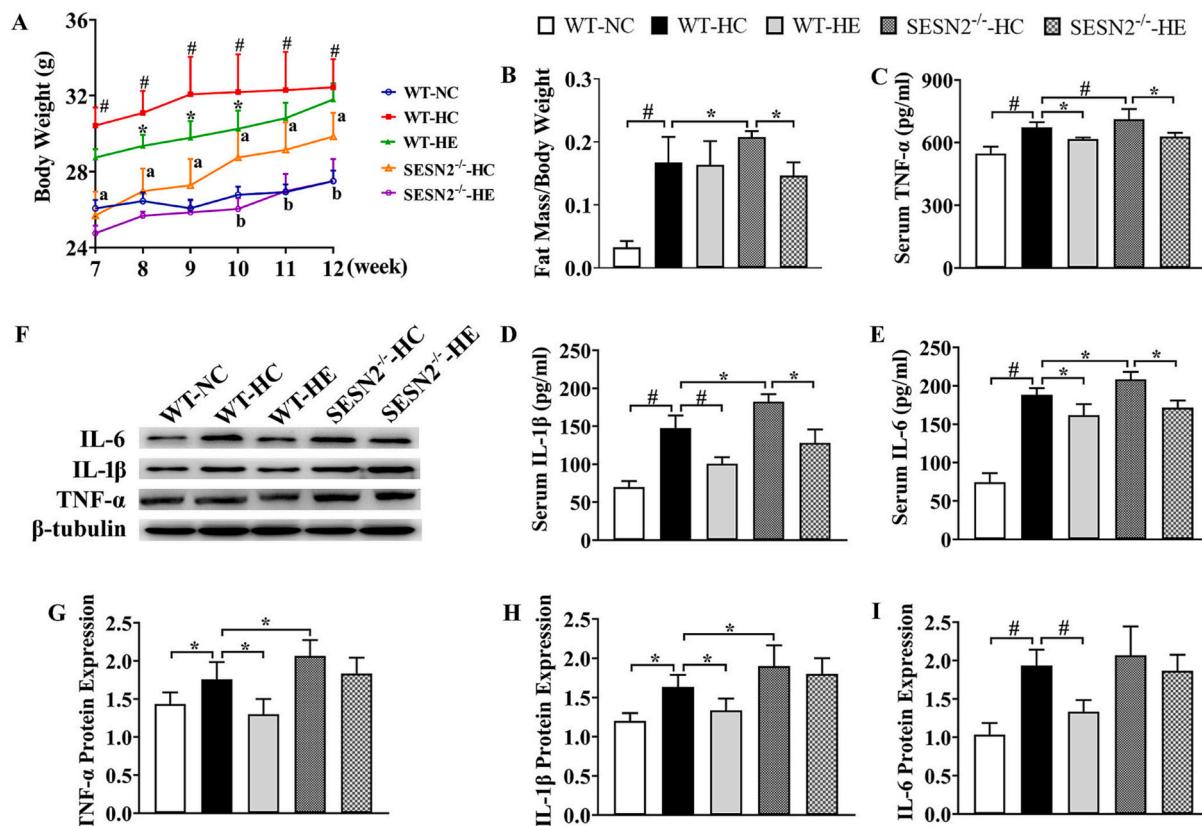
### 3.3. The interactions between SESNs and Nrf2 after HFD and aerobic exercise

Our data showed that SESNs directly bound to Nrf2 (Fig. 3E–H). Interestingly, we found that long-term HFD and aerobic exercise had no effect on the protein-protein interactions of SESN1 or SESN3 and Nrf2 (Fig. 3E, F, H), whereas long-term HFD promoted the binding of SESN2 and Nrf2 (Fig. 3E, G). These findings demonstrate that long-term HFD and aerobic exercise can notably affect the relationship of SESN2 and Nrf2, and therefore, we focused on the role of SESN2 in the following study. Given the important role of SESNs and Nrf2 in anti-inflammatory actions [28,29], we performed bioinformatics prediction using STRING database to reveal their relationships. Our results showed that SESN1 and SESN3 did not interact with Nrf2, whereas SESN2 did interact with Nrf2 (Fig. 3A–D). To further explore the relationship between SESNs and Nrf2, we performed an immunoprecipitation experiment following HFD and aerobic exercise treatments. Our data showed that SESNs were observed to coimmunoprecipitate with Nrf2, demonstrating SESNs can directly bind Nrf2 (Fig. 3E–H). Interestingly, we found that long-term

HFD and aerobic exercise had no effect on the proteins binding of SESN1 or SESN3 and Nrf2 (Fig. 3E, F, H), while long-term HFD led to an increase in the proteins binding of SESN2 and Nrf2 (Fig. 3E, G) in skeletal muscle. To investigate whether the interaction between SESN2 and Nrf2 is tissue-specific, we further performed the immunoprecipitation experiment in liver tissue. Similar to skeletal muscle, long-term HFD also led to a significant increase in the proteins binding of SESN2 and Nrf2 in liver (Supplementary Fig. 1A). These findings demonstrate that long-term HFD and aerobic exercise can notably affect the relationship of SESN2 and Nrf2, and therefore, we focus on the role of SESN2 in the following study.

### 3.4. SESN2 protein is required for exercise effects on Nrf2 pathway in HFD-fed mice

We further examined some proteins related to Nrf2 pathway in skeletal muscle of WT and SESN2<sup>-/-</sup> mice. Our data confirmed that SESN2 protein was completely knockout in SESN2<sup>-/-</sup> mice (Fig. 4A). Consistent with our previous studies [4,18], SESN2 protein notably



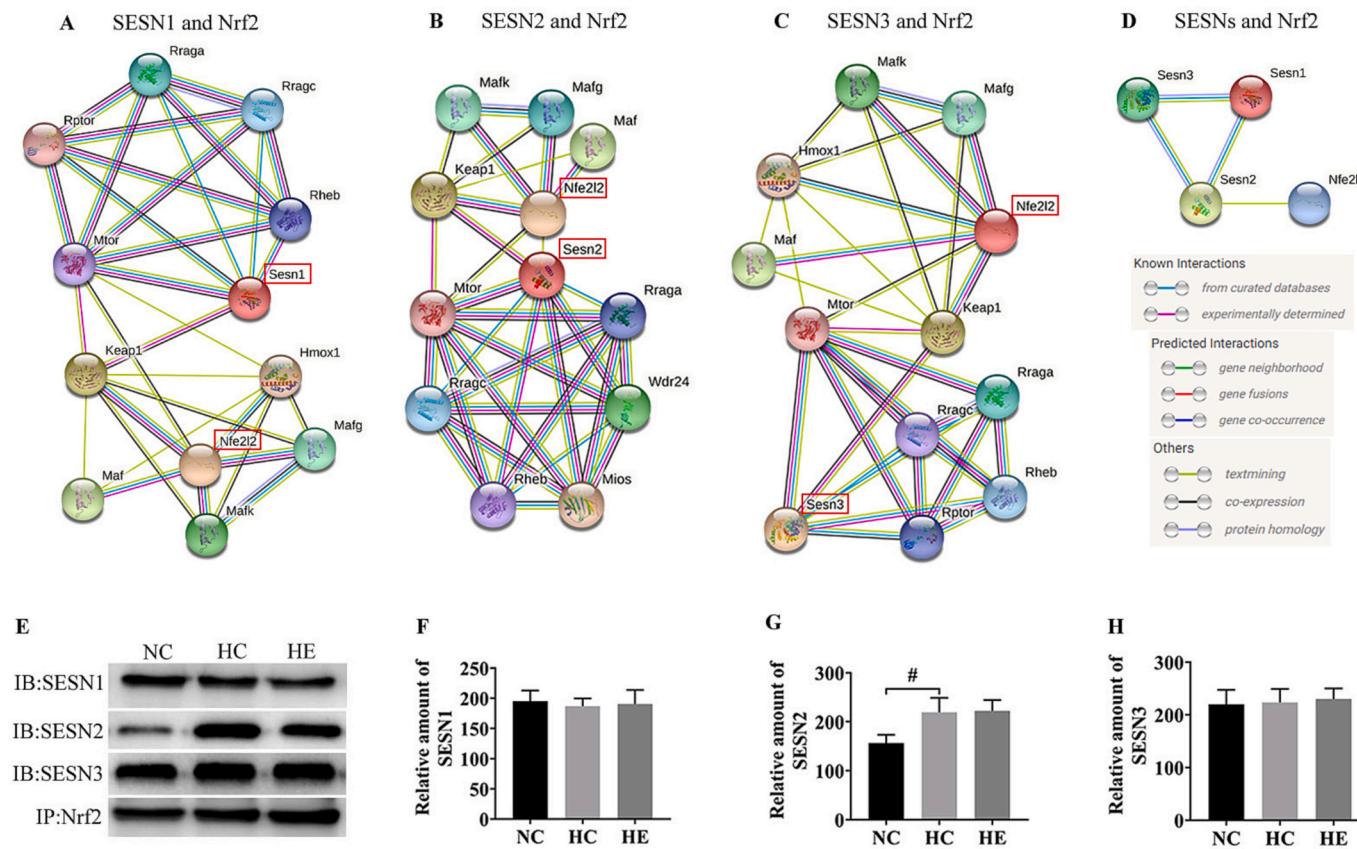
**Fig. 2.** Aerobic exercise ameliorated inflammation induced by HFD and SESN2 knockout. (A) Body weight was detected after 6-week exercise training. #  $P < 0.05$  WT-HC vs. WT-NC; \*  $P < 0.05$  WT-HE vs. WT-HC; a  $P < 0.05$  SESN2<sup>-/-</sup>-HC vs. WT-HC; b  $P < 0.05$  SESN2<sup>-/-</sup>-HE vs. SESN2<sup>-/-</sup>-HC. (B) Fat mass was evaluated and normalized to body weight. Values are means  $\pm$  SEM ( $n = 6$ ). \*  $P < 0.05$ ; #  $P < 0.01$ . (C–E) TNF- $\alpha$ , IL-1 $\beta$  and IL-6 were measured in serum after 6-week exercise training. Results were expressed as means  $\pm$  SEM ( $n = 6$ ). \*  $P < 0.05$ ; #  $P < 0.01$ . (F) Blotting and (G–I) quantitative analysis of proteins expression of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in the vastus lateralis muscle after aerobic exercise.  $\beta$ -tubulin was used as the internal control. Data are means  $\pm$  SEM ( $n = 6$ ). \*  $P < 0.05$ ; #  $P < 0.01$ . HFD, high-fat diet; WT, wild type; NC, normal chow; HC, high-fat diet chow; HE, high-fat diet chow plus exercise.

increase in response to the regular exercise in skeletal muscle of WT mice (Fig. 4A). Interestingly, long-term HFD also induced SESN2 protein increase in Fig. 4A, which may be a self-protection mechanism of organism. Moreover, this result is agreement with the reports of our and others [16,30]. Similar to SESN2 in WT mice, the variation trend of Nrf2 in nuclear was observed after HFD and regular exercise (Fig. 4B). However, compared with WT-HE group, Nrf2 protein decreased in skeletal muscle of SESN2<sup>-/-</sup>-HE mice, suggesting that increase of Nrf2 protein induced-exercise was inhibited after SESN2 knockout (Fig. 4B). Moreover, there was no significant difference of Nrf2 protein in SESN2<sup>-/-</sup>-HC and SESN2<sup>-/-</sup>-HE mice (Fig. 4B), indicating that SESN2 deficiency limited exercise effect on Nrf2 in the skeletal muscle of HFD-fed mice. We also detected their regulatory relationship in liver tissue. Similarly, Nrf2 protein also significantly increased after HFD and regular exercise in liver tissue of WT mice, and exercise-induced increase of Nrf2 protein was also restrained by SESN2 knockout (Supplementary Fig. 1B). These findings suggested that SESN2 is also essential for the exercise influences on the Nrf2 in liver tissue, which was consistent with the results in skeletal muscle (Fig. 4B). But it is not all the same in skeletal muscle and liver tissue. In skeletal muscle, there has no statistical difference of Nrf2 expression between WT-HC group and SESN2<sup>-/-</sup>-HC group (Fig. 4B); while SESN2 knockout led to a significant decrease of Nrf2 protein in liver (Supplementary Fig. 1B). Keap1 plays a guardian function on Nrf2 activity [21]. We found that cytoplasmic Keap1 protein significantly reduced in WT mice after long-term HFD and treadmill exercise, especially in exercise state (Fig. 4C). Although no significant difference of Keap1 protein has been observed between SESN2<sup>-/-</sup>-HC and SESN2<sup>-/-</sup>-HE mice, SESN2 deficiency did lead to a significant decrease of Keap1

protein when compared to WT mice (Fig. 4C). As predicted, HO-1, as a downstream of Nrf2, presented a similar trend of Nrf2 after long-term HFD and treadmill exercise both in WT and SESN2<sup>-/-</sup> mice (Fig. 4D). These results indicate that SESN2 protein is necessary for exercise effects on Nrf2 pathway in HFD-fed mice.

### 3.5. Nrf2 protein is required for SESN2 effects on inflammatory factors in PA+OA-treated C2C12 myotubes

To further assess the regulatory relationship between SESN2 and Nrf2, we examined the expression of inflammatory factors in PA+OA-treated C2C12 myotubes after SESN2 overexpression or Nrf2 inhibition administration. As shown in Fig. 5A, Nrf2 protein gradually declined in C2C12 myotubes that were incubated by 0, 5, 10 or 20  $\mu$ M ML-385 for 24 h, which confirmed the inhibitory effect of ML-385 on Nrf2. In addition, SESN2 protein is increased after Ad-Sesn2 treatment in C2C12 cells, suggesting that the adenovirus vector is effective in our study (Fig. 5B). We employed the classical method of the mixture of PA and OA to mimic dietary HFD to induce the lipid metabolism abnormalities in cell. The result of ORO staining showed 0.8 mM PA + 1.6 mM OA is the optimal concentration for the lipid accumulation in C2C12 myotubes, which resulted in concomitant increase in the number of lipid droplets (Fig. 5C). Similar to long-term HFD in vivo (Fig. 2), the inflammatory factors including IL-6, IL-1 $\beta$  and TNF- $\alpha$  were significantly increased in PA + OA-treated group when compared to the control group (Supplementary Fig. 1). The findings further revealed PA + OA treatment successfully induced lipid accumulation and inflammation in C2C12 myotubes. In addition, consistent with the trend of HFD in vivo (Fig. 4B



**Fig. 3.** The interactions between SESNs and Nrf2 after HFD and aerobic exercise in skeletal muscle. (A–D) The bioinformatics prediction of SESNs and Nrf2 in STRING database. (E) Blotting and (F–H) quantitative analysis of reciprocal immunoprecipitation of SESNs and Nrf2 after HFD-fed and exercise training treatment. Results are means  $\pm$  SEM ( $n = 6$ ). #  $P < 0.01$ . Nfe2l2 (also named Nrf2), nuclear factor erythroid derived 2-like 2; NC, normal chow; HC, high-fat diet chow; HE, high-fat diet plus exercise.

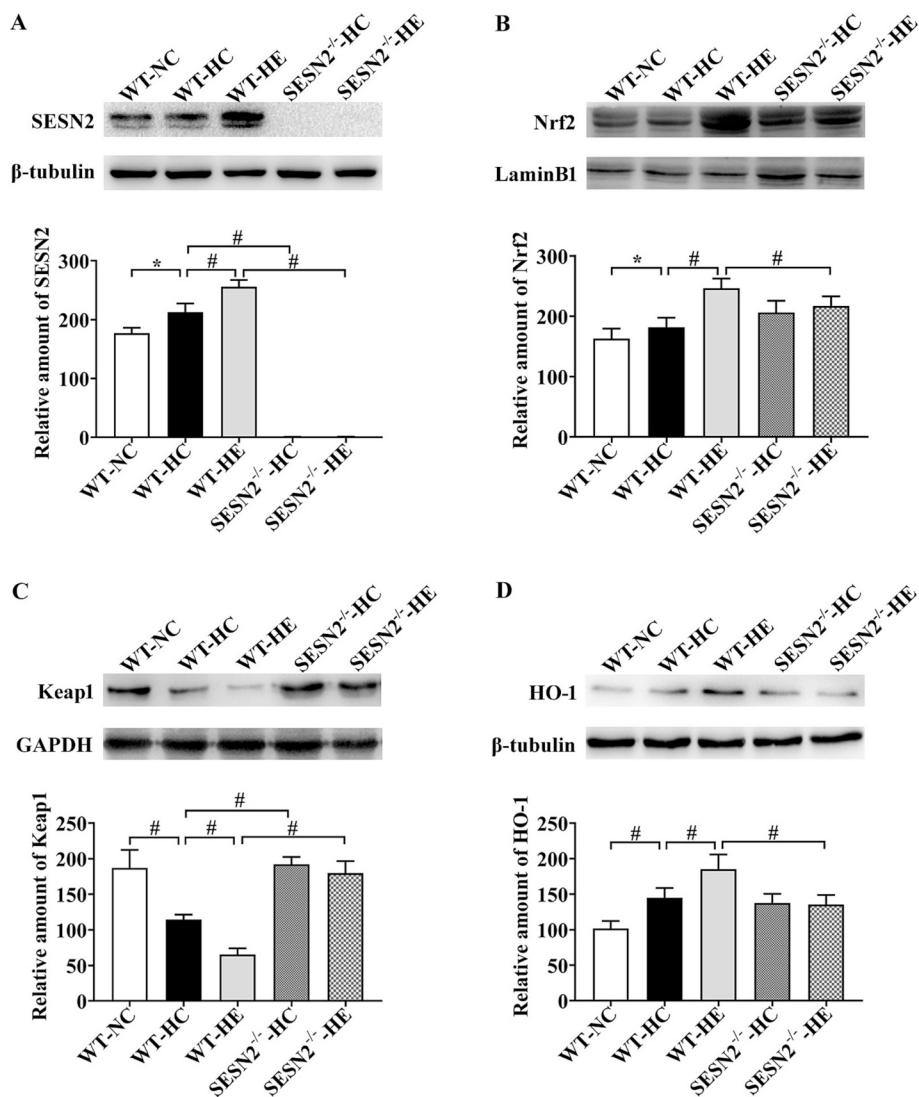
and D), we also observed that PA + OA treatment also led to a significant increase of Nrf2 and HO-1 protein expression (Supplementary Fig. 1). We observed Nrf2 protein significantly increased after Ad-SESN2 treatment in PA + OA-treated cell (Fig. 5D, E), while concomitant a decrease of IL-6, IL-1 $\beta$  and TNF- $\alpha$  proteins (Fig. 5D, F–H), demonstrating that SESN2 regulated Nrf2 expression and improved the inflammatory response. Conversely, ML-385 administration significantly increased the IL-6, IL-1 $\beta$  and TNF- $\alpha$  protein expression (Fig. 5D, E), which implies that inhibition of Nrf2 provoked an aggravated inflammatory response. As predicted, consistent with the trend of Nrf2, we observed that HO-1 protein also significantly increased after Ad-SESN2 treatment and also significantly decreased after ML-385 treatment in PA + OA-treated C2C12 myotubes (Fig. 5D, I). Moreover, compared to Ad-SESN2 group, the protein expression of IL-6, IL-1 $\beta$  and TNF- $\alpha$  was significantly increased in Ad-SESN2 + ML-385 group (Fig. 5D, F–H), suggesting that SESN2 plays the beneficial effects on inflammation via Nrf2 protein in C2C12 myotubes. In addition, there was no significant difference of these inflammatory factors between in ML-385 and Ad-SESN2 + ML-385 groups (Fig. 5D, F–H), directly indicating that Nrf2 protein is required for SESN2 effects on inflammation in PA + OA-treated C2C12 myotubes.

#### 4. Discussion

In this study, we found that the body weight of WT mice was significantly increased after 6-week HFD. To accurately assess the effect of genotype, we further calculated the ratio of body weight gain after HFD, which revealed that SESN2 deficiency exacerbated the body weight gain induced by HFD. Additionally, we discovered that SESN2 deficiency also enhanced the increase of trends in the fat mass, TC and

TG induced by HFD-fed. Our data demonstrated that SESN2 knockout aggravates the abnormality of body weight and serum lipid induced by HFD. This is consistent with our previous studies that SESN2 ablation led to obesity-related pathological changes, including TG accumulation, increased lipid deposition of liver and mitochondrial dysfunction in mice [4,16]. Moreover, Lee et al. also reported that systemic knockout of SESN2 in mice or dSesn in Drosophila resulted in obesity-induced mTORC1-S6K activation, glucose intolerance, insulin resistance, and hepatosteatosis [30,31], further validating the importance of SESN2 in the regulation of body weight and lipid metabolism.

Beside the above indicators, we further detected the inflammatory factors including TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in serum and skeletal muscle after HFD in WT mice. As expected, long-term HFD led to a significant increase of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in serum and skeletal muscle of WT mice, indicating that 6-week HFD can successfully induce chronic inflammatory response in mice. Indeed, excessive supply of energy such as Western diet in human or HFD in mice might be the main factor leading to chronic inflammation [8,9]. Moreover, our data showed that knockout of SESN2 stimulated the production of high levels of systemic inflammatory response induced by HFD. Similarly, SESN2 deficiency aggravated cardiac inflammatory response and damaged myocardium structure during ischemia and reperfusion [32]. In this study, WT and SESN2 $^{-/-}$  mice were subjected to a 6-week aerobic exercise after 6-week HFD treatment. In this study, WT and SESN2 $^{-/-}$  mice were subjected to 6 weeks of aerobic exercise after 6 weeks of HFD treatment. We found that, while aerobic exercise significantly reduced body weight and fat mass in both WT and SESN2 $^{-/-}$  mice, the beneficial responses of exercise were limited after SESN2 knockout. Notably, the results showed disparities in the anti-inflammatory effects of exercise training in serum

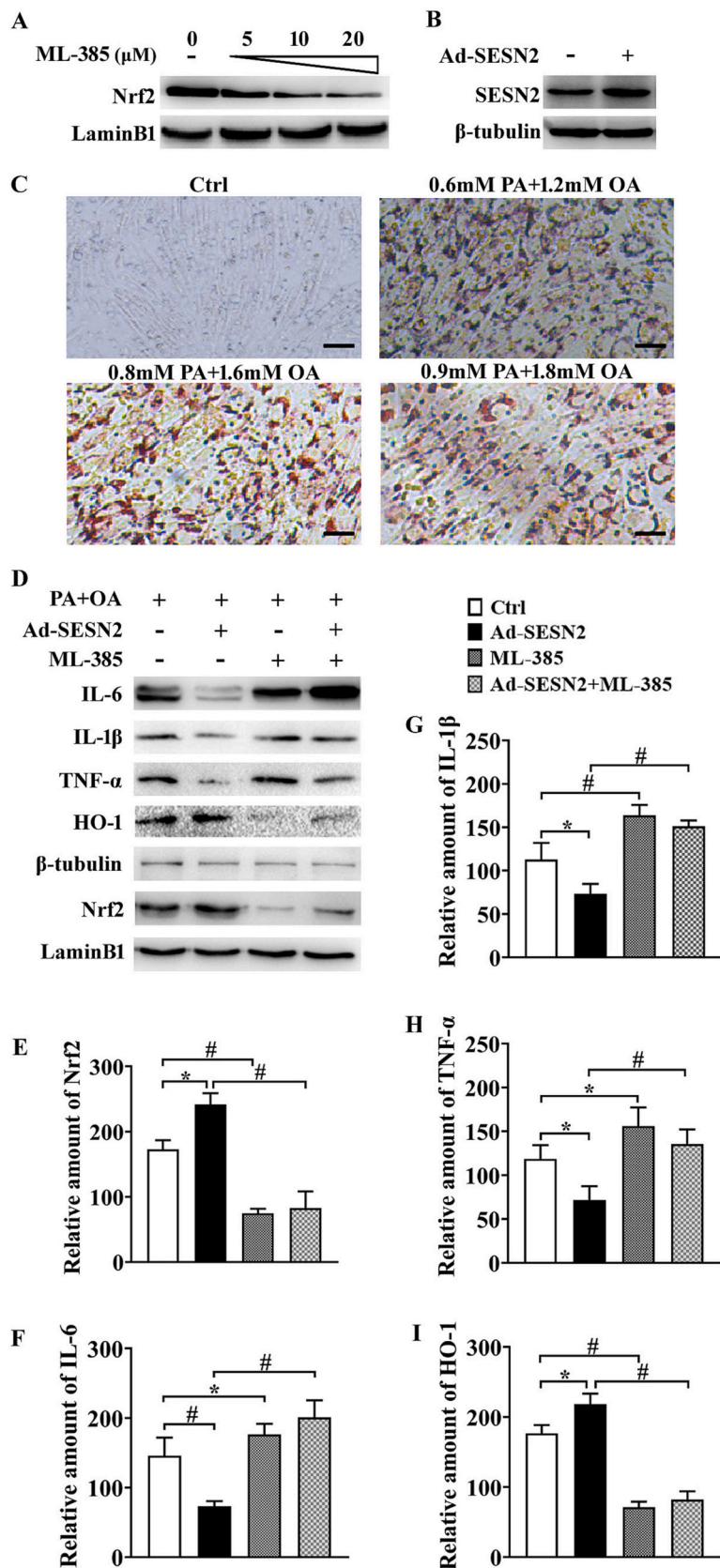


**Fig. 4.** SESN2 protein is required for exercise effects on Nrf2 pathway in HFD-fed mice. (A–D) SESN2, Nrf2, Keap1 and HO-1 protein expressions were detected after HFD-fed and exercise training treatment in the vastus lateralis muscle of WT and SESN2<sup>-/-</sup> mice. β-tubulin, LaminB1 or GAPDH served as the loading control. Values are means ± SEM ( $n = 6$ ). \*  $P < 0.05$ ; #  $P < 0.01$ . WT, wild type; NC, normal chow; HC, high-fat diet chow; HE, high-fat diet chow plus exercise.

and skeletal muscle of SESN2<sup>-/-</sup> mice. Molecular and cellular process of chronic inflammation is varied and depends on the type of inflamed cells and organ [33]. Exercise has been found to reduce chronic inflammation in obesity, while its underlying mechanisms remain unclear. The possible mechanisms include reducing the expressions of pro-inflammatory factors [34], enhancing the expressions of anti-inflammatory cytokines [34], inhibiting NLRP3 inflammasome activation [35] and reducing ROS production [36] in many tissues including blood and skeletal muscles. We found that aerobic exercise still decreased the content of TNF-α, IL-1β and IL-6 in the serum of SESN2<sup>-/-</sup> mice, suggesting that SESN2 is not essential for the regulation of aerobic exercise in ameliorating systemic inflammatory response. In blood, an inflammatory process includes a series of occasions with dilation of venules and arterioles, enhanced blood vessel permeability, and blood flow with percolation of leukocytes into the tissues [37]. In contrast, SESN2 is essential for exercise in reducing inflammatory response in skeletal muscles. Therefore, these results vary in different tissues due to the fact that skeletal muscle is able to rapidly adapt to drastic changes, while the changes of blood content is more complex and systematic during exercise [38,39]. In our study, the content of inflammatory factors in the serum represents the systemic and overall level of inflammation in the body. This may be the reason that SESN2 knockout did not

have many effects on blood, but did have many effects on skeletal muscles.

Growing evidence has demonstrated that protein-protein interactions are essential in various biological processes [39]. However, the interaction between SESN2 and Nrf2 remains largely unknown. We used the keyword SESN2/Nrf2 to search on Pubmed and found that there are few studies on SESN2/Nrf2 pathway currently. A recent study has suggested that Liraglutide ameliorated HFD-induced the increase in body weight, fat mass and lipids levels through upregulating SESN2-mediated Nrf2/HO-1 pathway [40]. SESN2/Nrf2 may play a cardioprotective role in cardiac dysfunction mice after 16-week HFD [41]. Furthermore, it has been reported that SESN2 is able to activate Nrf2 by promoting the Keap1 degradation, thus providing protection from oxidative liver damage [42]. Since SESNs and Nrf2 play a critical role in anti-inflammatory actions [28,29], we sought to gain an insight into their relationship using bioinformatics prediction and immunoprecipitation experiments. Our data showed that only SESN2, as opposed to SESN1 and SESN3, had a protein-protein interaction with Nrf2. Additionally, our results demonstrated that neither long-term HFD nor aerobic exercise had an effect on the proteins binding of SESN1 or SESN3 and Nrf2, while long-term HFD led to a significant increase in the proteins binding of SESN2 and Nrf2. Therefore, we are keen to explore the



**Fig. 5.** Nrf2 is required for SESN2 effects on inflammatory factors in PA + OA-treated C2C12 myotubes. (A) Blotting of Nrf2 protein in C2C12 myotubes that were incubated by 0, 5, 10 or 20  $\mu$ M ML-385 for 24 h. LaminB1 was used as the internal control. (B) Blotting of SESN2 protein in C2C12 myotubes that were infected with Ad-SESN2 for 48 h.  $\beta$ -tubulin was used as the internal control. (C) Representative images of C2C12 myotubes stained with oil red O ( $n = 3$ ). C2C12 myotubes were incubated with 0.6 mM PA + 1.2 mM OA, 0.8 mM PA + 1.6 mM OA or 0.9 mM PA + 1.8 mM OA for 24 h. Scale bars =100  $\mu$ m (D) blotting and (E–I) quantitative analysis of proteins expression of Nrf2, IL-6, IL-1 $\beta$ , TNF- $\alpha$ , and HO-1 in PA + OA-treated C2C12 myotubes. The PA + OA-treated C2C12 myotubes were infected with or without Ad-SESN2 for 48 h, plus with or without ML-385 (20  $\mu$ M) treatment for 24 h.  $\beta$ -tubulin or LaminB1 was used as the internal housekeeping protein control. All data are means  $\pm$  SEM ( $n = 6$ ). \*  $P < 0.05$ ; #  $P < 0.01$ . PA, palmitic acid; OA, oleic acid.

role of SESN2 and Nrf2 in further detail. Previous studies have indicated that physical exercise increases SESN2 protein in the skeletal muscle [4,16–19]. However, the studies regarding SESN2 expression in HFD-induced disease models were inconsistent. While the protein of SESN2 was increased in the liver of rats fed with HFD [43], our previous study verified that SESN2 protein significantly increased in the skeletal muscle of HFD-fed mice [16]. On the contrary, the expression of SESN2 was reduced in the liver of HFD-fed mice [44]. Nonetheless, the protective function of different SESNs against HFD related diseases remained consistent. Our present research revealed that SESN2 protein also notably increased in response to regular exercise in the skeletal muscle of WT mice. Moreover, HFD also induced an increase in SESN2 protein, which may be a self-protective mechanism of the organism. Furthermore, we evaluated certain proteins related to the Nrf2 pathway in the skeletal muscle of WT and SESN2<sup>-/-</sup> mice. Similarly, Nrf2 protein also significantly increased after long-term HFD and regular exercise in WT mice. This finding is in accordance with recent reports that exercise can augment the mRNA and protein expression of Nrf2 in skeletal muscle [25,26]. However, our data showed that SESN2 deficiency restrained the exercise effect on Keap1/Nrf2/HO-1 in HFD-fed mice, indicating that SESN2 protein is essential for the exercise influences on the Nrf2 pathway in HFD-fed mice.

Despite the progress made in understanding the role of SESN2 and Nrf2 in anti-inflammatory effects, the exact regulatory mechanism remains to be elucidated. Zhou et al. showed that luteolin mediated inhibition of Nrf2 led to decreased expression of sestrin2 mRNA in myocardial tissues of rats with ischemia/reperfusion injury [45]. While, Bae et al. found that overexpression of SESN2 activated Nrf2 by promoting the degradation of Keap1 and prevented oxidative liver damage [42]. It seems like that a positive feedback loop exists between SESN2 and Nrf2. Therefore, further studies are required to gain a more detailed understanding of the regulatory relationship between SESN2 and Nrf2 in models of chronic inflammation. To further investigate the connection between the two proteins, we examined the expression of inflammatory factors in PA + OA-treated C2C12 myotubes after SESN2 overexpression or Nrf2 inhibition. Our results showed that Nrf2 protein was significantly increased after Ad-Sesn2 treatment, while the proteins IL-6, IL-1 $\beta$ , and TNF- $\alpha$  were concomitantly reduced, indicating that overexpression of SESN2 promoted Nrf2 expression and improved the inflammatory response in cells. Additionally, the anti-inflammatory effects of SESN2 were limited by ML385, a specific Nrf2 inhibitor, thus confirming that Nrf2 protein is essential for SESN2's anti-inflammatory effects in PA + OA-treated C2C12 myotubes.

## 5. Conclusions

Our findings demonstrate that SESN2 knockout contributes to the abnormalities of body weight, serum lipid, and inflammation response caused by high-fat diet, and that aerobic exercise can mitigate these changes. Furthermore, we show that SESN2 protein is essential for exercise to reduce inflammation and regulate the Nrf2 pathway in the skeletal muscles of HFD-fed mice. Additionally, our results further confirm that Nrf2 is necessary for SESN2 to reduce inflammation in response to PA + OA treatment in vitro. Consequently, our study provides novel therapeutic strategies for exercise in chronic inflammation caused by HFD, and suggests that the SESN2/Nrf2 pathway may be part of the anti-inflammatory response to exercise for managing this condition.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbadi.2023.166792>.

## CRediT authorship contribution statement

SJL and HGL performed the experiments, analyzed and interpreted the data, and drafted the manuscript. YKZ performed the experiments, analyzed the data. SJL, HGL and HLS analyzed and interpreted the data.

LF and HLS critically revised the manuscript and were responsible for important intellectual content. All authors critically reviewed and approved the final version for publication.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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